

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Patent Application No. 09/463,890

Confirmation No. 6925

Applicant: Koszinowski et al.

Filed: April 28, 2000

TC/AU: 1636

Examiner: Fereydoun Ghotb Sajjadi

Docket No.: 203676 (Client Reference No. PA29186USFZSEhud)

Customer No.: 23460

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**DECLARATION UNDER 37 C.F.R. § 1.132 OF
ULRICH H. KOSZINOWSKI**

I, Ulrich H. Koszinowski, do hereby declare:

1. I am a co-inventor and co-owner of the above-referenced patent application. I am Professor of Virology and hold the Virology chair at the Ludwig-Maximilians-University of Munich. A copy of my *curriculum vitae* is enclosed herewith.

2. I have read Chartier et al., *J. Virol.*, 70: 4805-4810 (1996).

3. The method disclosed in the Chartier reference cannot be used to generate infectious virus genomes that are larger than an adenovirus genome (i.e., larger than about 36 kilobases). In particular, the technique of Chartier is not suitable for cloning herpes virus genomes, as demonstrated by the experiments described below, which were conducted at my direction.

4. As of 1997, the direct cloning of infectious herpesvirus genomes into plasmids *in vitro* was not possible and had not been reported. The inability to directly clone infectious

herpesvirus genomes into plasmids was due to the instability and the size of the herpesvirus genome in *E. coli*.

5. In 1997, viruses with relatively small genome sizes (up to ~20 kilobases) were generated using the well established methodology of molecular cloning in high copy number plasmids. However, these methods are not applicable to mammalian viruses with larger genomes, such as herpesviruses.

6. Like Chartier et al., my laboratory also used homologous recombination in *E. coli* to directly construct recombinant adenoviruses (see Ruzsics et al., *J. Virol.*, 80: 8100-8113 (2006), and Sirena et al., *Virology*, 343: 283-298 (2005)). However, this approach was successful only for adenovirus of species C and B. The approach was not successful for constructing infectious recombinant genomes of adenovirus species D.

7. Notably, attempts to maintain even a subgenomic fragment containing the Ad19a E3 region in standard high- and low-copy-number vectors in *E. coli* failed. We concluded that the instability of these viral genomes inhibits direct cloning. Indeed, sequence-derived instability of mammalian and viral DNAs or cDNAs, especially due to repeated sequences – a hallmark of herpesvirus genomes – has been identified as inhibiting construction of recombinant DNA by homologous recombination (see, e.g., Muyrers et al., *Methods Mol. Biol.*, 256: 107-121 (2004); Ruzsics et al., *J. Virol.*, 80: 8100-8113 (2006); Warming et al., *Nucleic Acids Res.*, 33: e36 (2005); Zhang et al., *Nat. Biotechnol.*, 18: 1314-1317 (2000)). Also, the construction of coronavirus (CoV) infectious clones *in vitro* was hampered by the size of the viral genome (around 30 kb) and the instability of plasmids carrying CoV replicase sequences in *E. coli*. Cloning infectious coronavirus genomes (30 kbp) was successful only using BAC technology (cloning in cells) applying standard BAC cloning techniques (see, e.g., Almazan et al., *Proc. Natl. Acad. Sci. USA*, 97: 5516-5521 (2000)). Thus these limitations reflect the properties of the genomes and the success of Chartier and colleagues was rather the exception than the rule.

8. Since direct *in vitro* cloning might represent a valuable alternative to cloning in cells, we attempted to use this technology to construct herpesvirus BACs directly, choosing MCMV genomes and KSHV genomes as representative genomes. These

experiments had to be terminated because of a lack of success. We found evidence that the mere size of the MCMV and KSHV genomes already inhibited the transposon-mediated genome marking for direct cloning *in vitro*. The data, still representing the state of the art in 2009, is set forth in Table 1 below. Therefore, the procedure disclosed in Messerle et al., *Proc. Natl. Acad. Sci. USA.*, 94(26): 14759-14763 (1997) and subject of the present application, was, at the time the present application was filed, and is still in 2009, the only possible method to construct infectious herpesvirus BACs (see, e.g., Brune et al., *Trends Genet.*, 16: 254-259 (2000); Ruzsics and Koszinowski, "Mutagenesis of the Cytomegalovirus Genome," In: *Human Cytomegalovirus*, T.E.Shenk and M.F.Stinski, eds. Berlin, Heidelberg: Springer, pp. 41-61 (2008)).

Table 1

Vector	High copy	Low copy	Low copy	BAC	BAC
Cloning approach	Traditional direct (ligation, transformation)	Traditional direct (ligation, transformation)	Homologous recombination direct	Homologous recombination direct	Homologous recombination indirect
SV40 (5 kb)	++	N.D.	N.D.	N.D.	N.D.
Adenovirus Species C (36 kbp)	--	+	++	++	++* N.D.**
Adenovirus Species D (36 kbp)	--	--	--	--	+* N.D.**
Coronavirus (30 kbp)	--	--	--	+	N.D
Herpesviruses (MCMV and KSHV) (120-230 kbp)	--	--	--	--	--* +** #

Key: ++: worked well, +: worked but was reported to reach the limit of the technique, --: did not work, N.D.: not done; *: *in vitro* transposon-mediated genome marking; **: *in vivo* transposon-mediated marking; #: method of the invention

9. I hereby declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: Munich Dec 6.2009



Ulrich H. Koszinowski

Attachments: Curriculum Vitae

CV Koszinowski

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Education/ Qualifications

1964 - 1969 Medicine, Universities of München and Göttingen
1970 Medical Assistant
1971 Medical Approbation and MD
1975 Venia legendi: Microbiology and Hygiene, Göttingen
1984 Professor, Virology and Immunology Faculty of
Biology, Univ. of Tübingen
1989 Medical specialization: microbiology & epidemiology

Positions held

1971 - 1976 Res. Assistant, Hygiene-Inst., University Göttingen
1976 - 1977 Res. Associate, University College, London
1978 - 1980 Heisenberg Award; Institute for Immunology &
Genetics, German Cancer Res. Center, Heidelberg
1980 - 1987 Director, Fed. Res. Inst. Animal Virus Dis. Tübingen
1987 - 1992 Chair Virology, University of Ulm,
1992 - 1996 Chair Virology; University of Heidelberg
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Honors

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